Effect of CSLM imaging rate on biofilms of *P. aeruginosa* and *S. aureus*

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Abstract

Biofilms are sessile communities of bacteria that can be found in an wide range of environments. Their inhabitants are phenotypically distinct from planktonic bacteria and are capable of forming complex, three-dimensional structures. Biofilms are studied using confocal scanning laser microscopy, or CSLM. This technique uses lasers and Novel Fluorescent Proteins (NFPs) to measure growth and structure formation of single- and multi-species biofilms *in situ* in three dimensions. We investigate the effects of slow and fast rates of image acquisition on mono- and co-cultures of biofilm forming bacteria: *Pseudomonas aeruginosa* and *Staphylococcus aureus*. After calculating growth rates and lag times, we find that fast scanning rates reduce the growth rate of *P. aeruginosa* in co-culture. Additionally, co-culture speeds up *P. aeruginosa* growth relative to monoculture when imaged at a slow rate, and fast scanning reverts co-culture growth to monoculture-like behavior. Additionally, a significant lag time is observed for *P. aeruginosa* grown in co-culture. The observed influence of confocal imaging rate on population dynamics should be considered in future studies to ensure accurate measurement of bacterial phenomena.

1 Background

Since Koch and Pasteur's discovery of bacteria in the mid-19th century, scientists have developed practices for culturing and studying these organisms in hopes of understanding them, fighting them off, and exploiting their unique properties. Growth media composed of well-mixed, motile bacteria, however, does not accurately mimic the natural environment of these cells. In other words, free-swimming planktonic bacteria are often poor models for studying natural behaviors [5]. Although there are a few real-world situations in which planktonic (free-swimming) forms prevail, such as sepsis and eye infection, most bacteria-driven problems occur because of another growth state: biofilms [19].

1.1 Overview of Biofilms

Biofilms are sessile (attached) groups of mainly bacteria adhered to each other and to a surface. They are surrounded by a self-produced matrix of extracellular polymeric substance (EPS) [8]. Biofilms may contain only one species, but they are more commonly found with a complex mix of multiple species or multiple kingdoms [12].

Bacterial cells can grow in one of two possible states: planktonic and sessile. The planktonic state is the main stage studied in todays microbiology labs, with a large number of cells well-mixed and suspended in media. The fast-growing planktonic cells, however, are rarely found in nature. In the lab, the cells are not subjected to stresses; as a result, they can constantly divide. Nature offers no such environment. More commonly, bacterial cells live in harsh conditions where survival, opposed to growth, is the main priority. In these situations, the sessile biofilm mode of growth is preferred.

The observed abundance of biofilms suggests that they offer other advantages to microbial inhabitants. Motivations for biofilm formation fall into four broad categories: improved defense, favorable habitat, community advantages, and default programming [5].

Biofilms typically form in response to cues from either nutrient availability or surface detection. Growth can occur at any interface; floating biofilms can even form at the interface between two fluids. Subsequent development follows five basic stages: initial attachment, irreversible attachment, maturation I, maturation II, and dispersion [21]. Initial attachment occurs via reversible van der Waals interactions between bacteria and the surface. These surfaces can be biotic or abiotic and are recognized by the cell through both specific and non-specific mechanisms. Some biofilms form upon recognition of a specific cell receptor, while others rely on mechanical surface sensing [3, 6]. More permanent attachment follows, allowing cells to adhere firmly

to the surface. At this time, bacteria increase their EPS production rate to chemically strengthen attachment. The biofilm develops in three-dimensionally in the first maturation phase as the number of cells increases. Regular cell division occurs, and additional planktonic bacteria may be recruited. During the second maturation phase, complex three-dimensional structures develop as the EPS builds vertically. Finally, mature biofilms undergo dispersal, periodically shedding planktonic bacteria to colonize new areas.

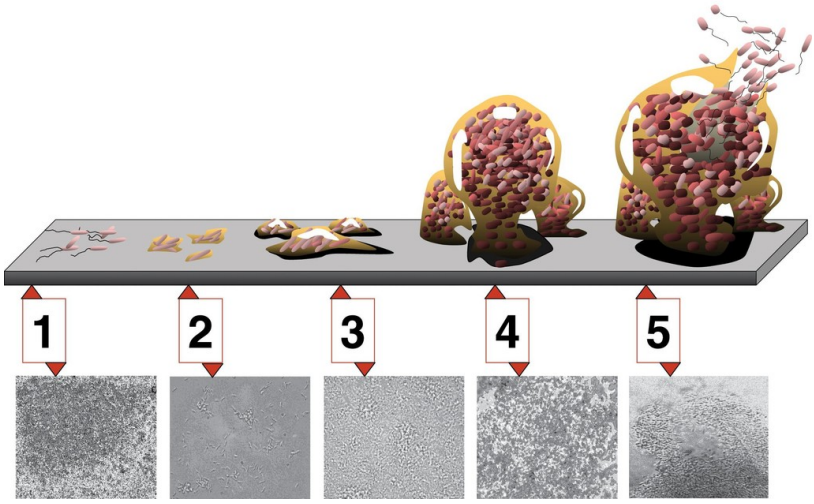

Figure 1: Image from www.boundless.com. The stages of biofilm growth.

1.2 Biofilm Properties

One of the defining characteristics of biofilms is the presence of EPS encasing the bacterial cells. EPS is a polymeric conglomeration of mainly polysaccharides, but often contains extracellular DNA, proteins, nutrients, and other debris from the environment [8]. The distribution of these materials will be inhomogeneous across the biofilm. The EPS acts as a glue to keep the bacteria together while allowing the development of strong, complex, three-dimensional constructs. For example, where a rapidly dividing clump of cells are expected to form a pyramid structure (large base of support), biofilms often develop mushroom-shaped structures (small base of support). Also observed are channels that run deep into thick biofilms, allowing nutrients and water to be carried to parts of the biofilm that would otherwise be inaccessible [5]. It is possible, however, that these structures are an artifact of the equipment used to study biofilms.

In addition to providing physical support, EPS keeps the biofilm from homogenizing chemically [10]. This effect is compounded by spatial structure and density of the biofilm. This allows the biofilm to condition the microenvironment of the bacteria

by localizing enzyme concentrations, pH, metabolite concentrations, charge, signaling molecules, etc., while controlling the diffusion barriers for each. Not only do diffusion barriers keep toxins from penetrating the biofilm, they also keep important, reusable cellular products close to the cells. For example, enzymes concentrations can be locally elevated so that the cells can use focus their resources elsewhere. The chemical control allows the biofilm to create optimal conditions despite unfavorable conditions outside of the biofilm [12]. The bacteria respond differently to varying microenvironments by adjusting metabolism and growth. The differential responses of the cells to specific microenvironments further complicate the inhomogeneity of the biofilm, eventually giving rise to specialized cell functions within the biofilm [2]. These chemical differences account for the ability of anaerobic bacteria to survive in aerobic conditions; oxygen is consumed by aerobic bacteria or sequestered chemically to other parts of the biofilm [15].

Apart from controlling the microenvironment of the bacteria, the EPS encasing also protects the biofilm from outside stresses. Strong, EPS-cased structures are able to withstand shear stresses of flow or physical scraping [8]. Because of the control of diffusion barriers, biofilms are able to withstand treatments that could kill off planktonic bacteria. Chemicals such as antibiotics or detergents are unable to penetrate into the biofilm. In addition, the bacteria on the outer layers act as a wall for the inner layers, preventing any unwanted chemical activity inside of the biofilm [22]. In fact, the biofilm is able to increase antibiotic resistance by up to 1000 times compared to their free-swimming counterparts [23]. This effect is also due in part to phenotypic changes in gene expression [22]. In a biological setting, the EPS encasing prevents host macrophages from engulfing microbial cells. Moreover, the continual failed activity of the macrophages around the biofilm can damage the host organisms tissues [11].

The adhesion of planktonic bacteria to a surface triggers the activation of a large set of genes due to the expression of the σ factor [13]. These genes cause the bacteria to become clearly phenotypically different from the planktonic state. In fact, these differences are visible when a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel is run to separate proteins of different masses. These gels show stark differences of the expressed proteins between planktonic and biofilm bacteria. Furthermore, the ability of biofilms to create microenvironments result in different gene expression in different regions to suit the local necessities of the biofilm [8]. The differences in phenotype can be further augmented through the extracellular DNA present in the EPS. Like the localization of enzymes, extracellular DNA such as plasmids are localized and therefore easily transferable from cell to cell. The EPS keeps the plasmid in a favorable condition while also keeping the cells and plasmids close physically, increasing the chances of a successful transformation [20]. Commonly called horizontal gene transfer, this promotes genetic diversity in the biofilm across species and allows the biofilm to have a pool of genetic material to pull from when

subjected to a new environment. For example, a plasmid with antibiotic resistance could be transferred more rapidly in a biofilm than between planktonic cells. Thus, even the distribution of phenotypes across a biofilm are inhomogeneous. This aids in the specialization of cell function. However, the phenotypic differentiation that the biofilm bacteria undergo is not permanent; when placed in a new environment, the bacteria can respond and change its gene expression.

Once a certain density of cells is reached, the biofilms are able to communicate through quorum sensing [9]. Quorum sensing is bacterial cell-to-cell communication commonly used so that the entire biofilm can respond to an external stimulus on a localized of the biofilm. After a stimulus, bacterial cells will release chemical signals called autoinducers that act as a transcription factor by directly regulating the gene expression of other quorum sensing bacterial cells. Autoinducers increase in concentration proportionally to the cell density. Quorum sensing allows individual bacteria to react to changing population densities as a unit. Some effects of the release of autoinducers include the shedding of planktonic cells to colonize new bacteria or the recruitment of planktonic bacteria into the biofilm. Because of the aforementioned channels and heterogeneities (both in cell phenotype and microenvironment properties), the autoinducers will travel to specific locations, allowing the biofilm to respond to stimuli in ways that planktonic cells cannot.

The differences in cell phenotype and behavior, along with specialization of roles, culminate in a complex microbial community that exhibits metabolic cooperation, the ability to respond to external stimuli, primitive homeostasis, and a simple circulatory system. Biofilms can even be considered to behave like primitive multicellular organisms despite being composed of individual bacteria capable of life on their own. In fact, biofilms act so much like eukaryotic organisms that bacteria can undergo a process similar to apoptosis where individual bacteria trigger cell death [12]. The differences in phenotype between bacterial cells within the same biofilm demonstrate the genotypic flexibility that bacteria have and how being in a biofilm unlocks the full potential of their genotypic regulation. It can be said that the widespread success of bacteria in so many rapidly changing environments can be due to their flexibility in genetic expression, particularly within a biofilm.

1.3 Real World Significance

Because of their flexibility and ability to survive harsh conditions, biofilms are present in many environments, both natural and industrial. The development of unwanted biofilms in industrial situations can cause significant complications. In in the US healthcare system alone, biofilms cost billions of dollars annually because of biofilms growing on both host tissues and implanted devices [16]. Since biofilms are able to grow on any surface in hospitals including implants, wound dressings, and other

medical devices, they are the main cause of nosocomial (hospital-acquired) infections. Additionally, biofilms can act as a reservoir for pathogenic bacteria where they continually release disease-causing bacteria, even after the disease is treated.

In an industrial setting, biofilm development quickens the corrosion of metals and clogs pipes. Equipment becomes damaged and unusable much faster than expected. In addition to damaging the machinery itself, biofilms can contaminate products through both virulent planktonic cells and the release of metabolic product waste [18].

However, this is not to say that biofilms only cause detrimental effects. Biofilms are vital to many symbiotic relationships, such as the nitrogen-fixing biofilms located in plant roots. Additionally, biofilms layer the inside of human intestines, aiding with digestion of food.

Industrially, biofilms are used in wastewater treatment. While rocks and sand can filter out solid pieces of debris, biofilms are able to transform chemicals that are dissolved in the wastewater. These biofilms can be controlled to specifically filter out certain organic chemicals found in different wastewater origins [4]. The ability of bacteria to convert hydrocarbons also becomes useful in treating oil spills. A certain group of hydrocarbonoclastic bacteria are effective in converting petroleum to other, non-toxic chemicals [14]. Microbial fuel cells can be used to generate electrical current from organic materials and other degradable biomasses [3].

1.4 Common biofilm-producing bacteria

Particularly, biofilms are responsible for the chronic lung infections found in patients suffering from cystic fibrosis (CF) [7]. *Pseudomonas aeruginosa* commonly forms a monospecies biofilm in chronic lung infections of CF patients. As expected, these biofilms are incredibly resistant to antibiotic and the bodys innate immune responses, causing chronic inflammation from continual activity from the bodys immune system. *P. aeruginosa* are motile, aerobic, rod-shaped bacteria that are considered a model organism for studying biofilms.

Nosocomial infections are often caused by *Staphylococcus aureus*, a spherical bacteria. They are commonly found on skin and mucus surfaces of humans and other animals. However, the presence of *S. aureus* does not always imply a virulent infection. One strain of *S. aureus*, MRSA, has emerged as an antibiotic resistant strain, causing large concern over possible treatments [1].

1.5 Laboratory Techniques

In the lab, biofilms are grown in a flow cell. Flow cells are similar to regular slides used in analyzing planktonic microbial cells, but contain chambers that allow media to over the biofilm. This keeps the bacteria alive by giving them a surface to adhere to, an environment to form a biofilm, and a constant supply of nutrients. Regular slides do not provide these three conditions. Each flow cell has three separate chambers that can grow three different biofilms. The basic setup is a closed system from which media is pumped through the flow cell and into a waste container. After inoculating the flow cell chambers with rapidly dividing planktonic bacteria, the cells are given an hour to attach to the coverslip of the flow cell before flow is initiated to ensure that they are receiving the proper nutrients. The flow cells parameters can be manipulated greatly to study various factors. These parameters include the species of bacteria in the chambers, strains of bacteria, the flow rate, the concentration of sugars, the presence of antibiotic, temperature, etc.

Microscopy has now opened the doors to allow for analytical imaging. In particular, confocal scanning laser microscopy (CSLM) is a digital imaging device that allows the analysis of living biofilms in situ. CSLM is a microscopy technique that is able to select for depth while taking images. The images are acquired point-by-point and sent to the computer, where they are reconstructed. This allows for the creation of three dimensional models of topologically complex subjects, including the imaging of interior structures. By using a focused laser beam, biological subjects with fluorescent markers can be studied. CSLM works by detecting the light from these fluorescent markers. An excitation laser is sent through the aperture and focused by an objective lens into a small focal plane. Some scattered light and emitted fluorescent light pass again through the objective lens where the light is refocused. This refocused light is then separated by a beam splitter, directing the light onto another aperture with a filter that blocks the original excitation wavelength. Thus, only the emitted fluorescent wavelengths emitted by the bacteria are able to bypass the second aperture. These emitted wavelengths are read by the computer through a light detection device such as a photomultiplier tube (PMT) that amplifies the signal. Once the bacteria are found, the focal plane can be adjusted in the z direction so that a series of images can be constructed at different heights. These images together form a three-dimensional image called a z-stack. With respect to biofilms, a sequence of z stacks of a certain region can be taken to watch the development of a volume of biofilm over time. By using transformed bacteria with a specifically placed biomarker such as GFP, the expression of certain genes or growth of bacteria can be monitored over time. The confocal microscope also has a controller that moves the objective in the xy-plane so that all three chambers can be observed sequentially [17].

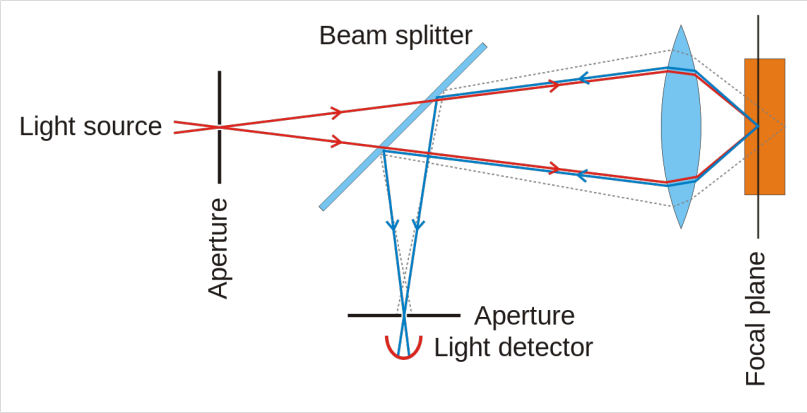

Figure 2: Image from www.medindia.com. Visual representation of CSLM beam path. The focal plane can be adjusted for height.

1.6 Purpose

Though confocal microscopy is useful in determining the three-dimensional structures of biofilms, increased laser exposure could affect their growth. This effect can be quantified by analyzing growth curves of bacteria subjected under different imaging rates on a confocal microscope. In addition, we examine whether scanning perturbs grown in co-cultures and monocultures.

2 Materials and Methods

2.1 Bacterial Strains

P. aeruginosa strain PA01 rsal::gfp is the laboratory strain commonly used when studying the bacteria. The specific strain used has been transformed so that it produces green fluorescent protein (GFP) when producing a protein. The fluorescence can be picked up by the confocal microscope on the CFP or GFP channel. We used the CFP channel because the GFP channel was more susceptible to unwanted fluorescence. PA14 only produces Pel, while PA01 produces both Pel and Psl. Pel and Psl are both polysaccharides that are commonly used in biofilm formation. PA14 is more virulent than PA01. Various other genetic differences are minor and are not expected to affect the experiment significantly. These strains have increased antibiotic resistance to carbenicillin because of genes on the fluorescence plasmid.

The strain of*S. aureus* used is MN8. pJY209 yfp, refers to the plasmid used for production yellow fluorescent protein (YFP) to be read in by the YFP channel of the confocal. S. aureus strains have increased antibiotic resistance to erythromycin due to genes on the fluorescence plasmid.

2.2 Procedure

2.2.1 Initial Growth

We grew up PA01, PA14, and pJY209 separately overnight. They were then diluted and allowed to grow until they were in an exponential growth phase. This was monitored by checking the optical density (OD) of the planktonic bacteria until OD of 0.3, which is known to be in exponential phase. The bacteria were then mixed and diluted to obtain a total OD of 0.0015. The flow cell was then inoculated with 1 mL of bacteria mixture using a syringe and needle.

OD involves measuring the percentage of a certain wavelength of light that is absorbed by an aqueous solution. The more particulates are present in the solution, the higher the OD. OD measurements are a quick way of measuring the growth of planktonic cells without disturbing growth.

Often, *P. aeruginosa* and *S. aureus* form co-culture biofilms together in nature. So, we made 5 separate cases of monoculture and co-culture bacterial biofilms. Three monocultures were created: PA01, PA14, and pJY209. Two co-cultures were created: PA01 with pJY209, and PA14 with pJY209.

2.2.2 Imaging

After the bacteria were inoculated, the bacteria were given an hour for attachment before beginning the flow of the media. During this time, regions of attached bacteria were found. These were to be the locations upon which biofilm growth would be observed. The different locations were queued so that the confocal microscope could move to each location and take z-stacks over time.

Each of these biofilms were exposed to varying image acquisition speeds. The varying acquisition speeds changed the amount of laser exposure each biofilm received. As images were taken, we could measure the biomass growth of the biofilm to determine how well the biofilm was growing under varying imaging rates. The imaging rates used were: 2, 2.3, 3.4, 4, 60, 61.2, 62, and 67 minutes/frame. These were grouped in two general categories: slow and fast scans. Imaging rates 2-4 were grouped as fast rates while rates 60-67 were grouped as slow rates.

2.2.3 Voxel Counting

Biomass growth was determined through voxel counting, a count of the number of bright voxels at a point in time. A voxel is a volume pixel, a value of a three-dimensional grid. As biofilms develop, the number of cells expressing Novel Fluorescent Proteins (NFPs) and the amount of expression of NFPs increase. Bacteria that are fluorescing are read into the computer as a collection of bright voxels. Because

confocal scanning gathers data in three directions, a program can scan through all of the voxels in a z-stack, recording the number of bright voxels. Over time, the number of bright voxels will increase according to the growth of the biofilm. Thus, voxel counts are a proxy for biomass growth.

Eventually, the bacteria cover the entirety of the field of view, forming a mat. After a mat forms at about 8 hours, the bacteria quickly become dim. Dimly lit bacteria do not indicate that the bacteria are dying or growing badly; it means that they are no longer expressing the NFP plasmid genes or the protein isnt folding properly due to low oxygen levels. Data was collected from the first eight hours of growth since the experiment centered on initial biofilm attachment and development.

Bright voxels are counted if the brightness of the signal is above a certain threshold. The confocal writes the brightness of the voxel as a function of the intensity of the incoming signal. A threshold is established by adjusting the brightness threshold on the image viewing program Fiji. The z-stacks are read into the program, and the brightness adjusted until an appropriate amount of noise is excluded while bacteria are included. These thresholds were kept constant for each strain for all experiments.

2.2.4 Growth Curves

Plotting voxel counts over time gives a growth curve for each species individually. Each growth curve was normalized so that the initial value was 1. If $N(t)$ represents the number of bright pixels as a function of time, data was actually plotted as $N(t)/N_0$, where N_0 represents the number of bright pixels at time $t = 0$. The bacteria used expressed different NFPs, allowing for selection of a specific bacteria when imaging. The PA01 and PA14 respond to the CFP laser and do not respond to the YFP laser. Similarly, pJY209 responds to the YFP laser. Thus, by imaging using two different lasers, separate growth curves for each species can be generated while keeping the biofilm physically unperturbed. The plots for the growth for each species reveal individual behaviors that can be analyzed.

It is expected that the bacteria exhibit some sort of exponential growth modelled by the function $y = Ae^{(t-s)/\tau}$ with A being approximately 1. Here, t is the time, s is some time delay of growth, and $1/\tau$ is a growth rate. The higher the $1/\tau$ value, the faster the biofilm growth. The delay s describes a lagging phase in the bacterial growth curves where no net growth or bacterial death was occurring.

2.2.5 Log-Linear Plots and Fitting

Some plots had multiple exponential phases such that the plots couldnt be easily modelled by a combination of exponential functions in order to calculate their growth rates. To easily see the multiple exponential phases, the voxels counts were plotted

against on a logarithmic scale such that the exponential phases of growth would appear linear. Regimes of growth that were not exponential in nature were not linear. Using this method, there were clear cusps where the growth rates changed. This allowed the grouping of data into time sections, isolating specific exponential phases.

The slopes of these linear-looking regimes on the logarithmic plots give the growth rates ($1/\tau$) of that exponential growth regime. The linear model is of the form $ln(y) = \frac{1}{\tau}t + ln(A)$. Because A is approximately 1, $ln(A) \approx 1$.

Additionally, lag times could be established for each growth curve. The areas with negative slope or a slope of zero indicated that the bacteria did not immediately begin exponential growth, but had some delay. These values were found simply by finding the time at which the first exponential phase began. Biofilms that experienced no lag time were given a lag time of 0. Thus, $1/\tau$ growth rates and lag times were quantified for each growth phase for each bacterial strain.

3 Results

Bacteria selectively did not grow in the scanned field of view. This effect was seen in two ways. The first, in later stages of biofilm growth, a large number of bacteria migrated from outside of the field of view, almost as if they were spilling over from an area of high density to low density. Second, after the biofilm was fully developed, images of the corners of the field of view showed stark differences between the development of the biofilm inside and outside of the field of view. It is possible that although confocal microscopy is useful in reconstructing three dimensional structures, the continued use of a laser on a biofilm can have negative effects on its growth.

Though this effect was qualitatively seen, we conducted quantitative analyses of the data. The table below shows the number of experiments conducted for each situation.

Rate	PA01	PA14	pJY209	PA01 & pJY209	PA14 & pJY209
Fast	3	2	2	4	3
Slow	6	0	4	12	4

Experimental data yielded growth curves, as stated above. Typical growth curves are shown below.

The growth rates and lag time averages and standard deviations are summarized in the table below.

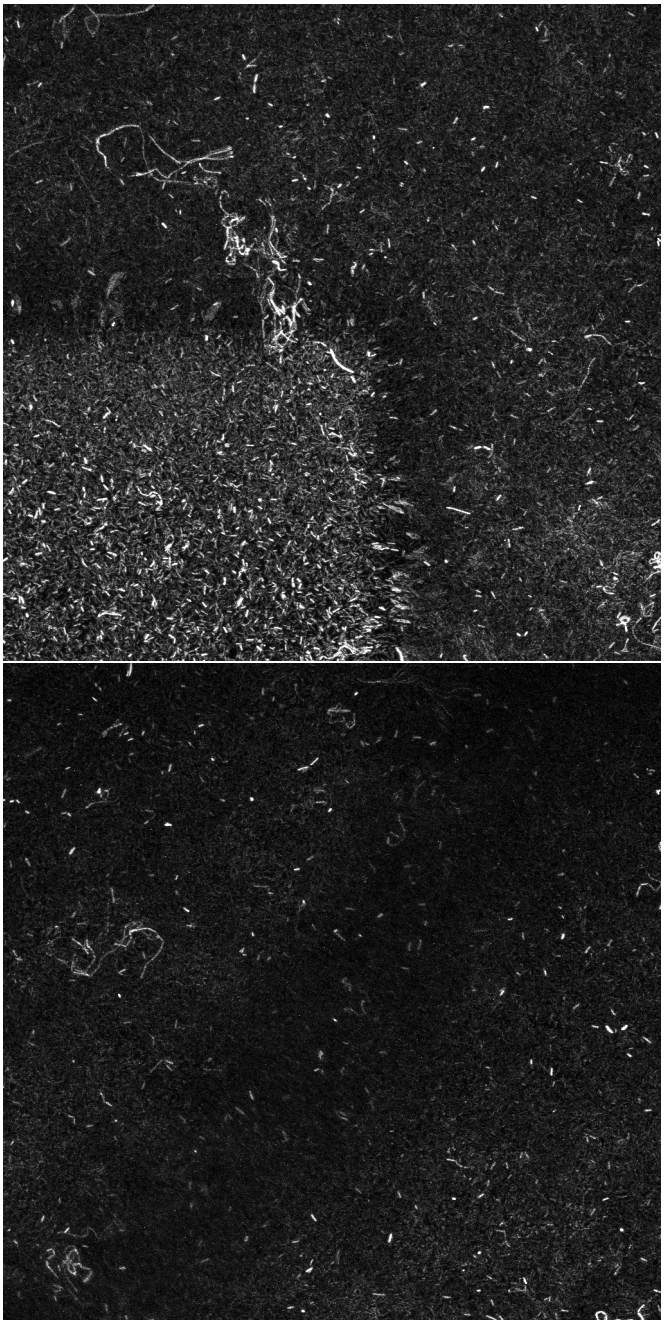

Figure 3: Images taken using CSLM. The corner of the field of view under fast scanning is shown in the top picture. The corner of the field of view under slow scanning is shown in the bottom picture. A clear difference in biofilm development between the imaging and non-imaging area can be seen in fast scans. However, this effect is not seen in the slow imaging image.

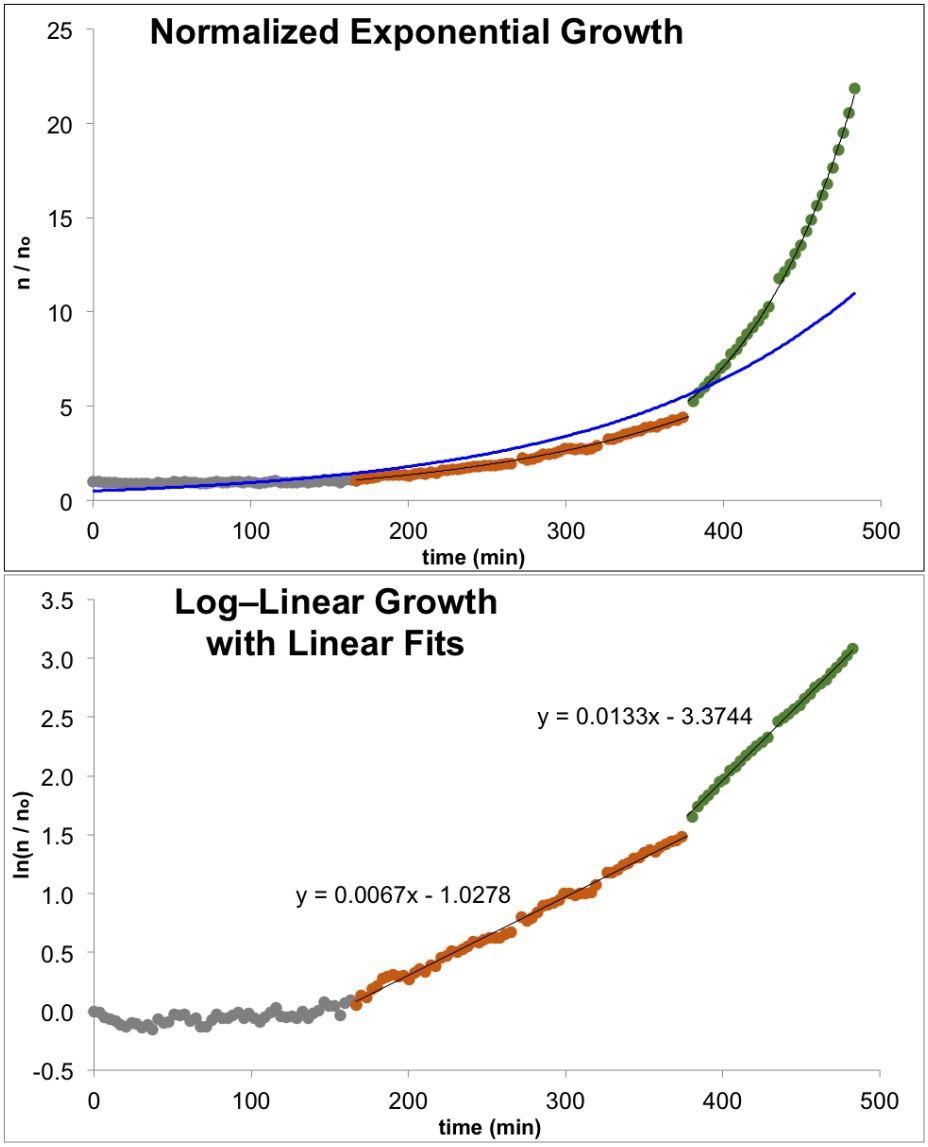

Figure 4: An exponential fit, even with a horizontal shift, improperly fits the data as shown in blue in the top graph. This is because of a second phase of exponential growth. Plotting the growth curves on a log-linear plot reveals clearly the different exponential phases based on different slopes of the linear fits. The first exponential phase is shown in orange, the second exponential phase is shown in green, and the lag phase is shown in grey. The fit values for the log-linear plot are shown. The non-linear activity before the growth is not modelled by exponential growth. Note that because there is a lag time, the intercepts are not at y=0.

Growth Rate (per minute)	Fast		Slow	
	Average	Std. deviation	Average	Std. deviation
PA01 mono	0.0080	0.0023	0.0066	0.0017
PA01 co	0.0062	0.0006	0.0117	00017
PA14 mono	0.0054	0 .0026	-	-
PA14 co	0.0047	0.0026	0.0053	0.0040
pJY209 mono	0.0083	00.0018	0.0085	0.0028
pJY209 co-PA01	0.0094	0.0043	0.0118	0.0024
pJY209 co-PA14	0.0099	0 .0020	0.0133	0.0012

Lag Time (minutes)	Fast		Slow	
	Average	Std. deviation	Average	Std. deviation
PA01 mono	91.3	53.5	39.5	28.4
PA01 co	118.0	18 .0	94.9	64.0
PA14 mono	214.0	65.0	-	-
PA14 co	190.0	173.0	288.0	66.0
pJY209 mono	0.0	0.0	30.0	60.0
pJY209 co-PA01	0.0	0.0	5.2	17.9
pJY209 co-PA14	77.0	100.0	30.0	60.0

Sets of data were compared to each other to see if there were significant effects of certain characteristics. These data sets were compared using a two-tailed t-test, resulting in p values indicating significance of results. An α-level of 0.05 was set. The p values are summarized in the tables below. Significant p values below the threshold are bold. PA14 is excluded due to lack of data.

PA01 Growth	FAST vs SLOW		MONO vs CO	
	MONO	0.4920	FAST	0.3857
	CO	**0.0081**	SLOW	**0.0008**

pJY209 Growth	FAST vs SLOW		MONO vs CO	
	MONO	0.9319	FAST	0.7934
	CO	0.5655	SLOW	0.0874

PA01 Lag		FAST vs SLOW		MONO vs CO
	MONO	0.3019	FAST	0.5735
	CO	0.4377	SLOW	**0.0351**

pJY209 Lag		FAST vs SLOW		MONO vs CO
	MONO	0.3910	FAST	1.0000
	CO	0.3388	SLOW	0.4713

4 Discussion

The slow imaging rate can be considered a quasi-native state in which there isnt much perturbation from the laser. However, it cannot be said that slow imaging rates do not affect biofilm growth; it simply affects the biofilm growth less. Growth rates taken at this imaging speed can be considered the base growth for this experiment. Likewise, fast imaging rates can be considered the non-native state. There are thus four states that can occur: quasi-native monoculture, quasi-native co-culture, non-native monoculture, and non-native co-culture. Due to inappropriately sized data sets, PA14 is generally excluded from the analysis. However, average values and p-values are still calculated and shown in tables.

4.1 Conclusions

4.1.1 PA01

PA01 monocultures growth rate is not affected by imaging rate ($p = 0.4920$).

A co-culture with PA01 and pJY209 in the quasi-native state increases the rate of growth rate compared to the monoculture ($p = 0.0008$).

Imaging the co-culture in the non-native state reverts the growth rate back to quasi-native monoculture growth rates ($p = 0.0081$). However, co-cultures and monocultures have no difference in PA01 growth rates in the non-native state.

The only significant change in lag time is an increase in PA01 lag time in co-culture compared to monoculture when imaged in the quasi-native state ($p = 0.0351$).

4.1.2 pJY209

pJY209 monocultures growth rate is not affected by imaging rate ($p = 0.9319$).

The presence of PA01 in a pJY209 biofilm does not affect the pJY209 growth rate ($p = 0.0875$). However, this statistic could become significant if more data is collected.

Imaging the pJY209 and PA01 co-culture in the non-native state does not affect growth rate ($p = 0.7934$).

Finally, imaging rate has no effect on pJY209 growth rates between monoculture and co-culture ($p = 0.5655$).

4.1.3 Possible causes

The increased growth and increased lag time of PA01 in co-culture than monoculture in the quasi-native state indicates some sort of change in the environment when pJY209 is present. This could be different quorum sensing molecules or pJY209 peptidoglycan affecting the PA01 growth. It is possible that this lag time is due to the PA01 shifting its gene expression to a phenotype that allows faster growth.

PA01 in non-native monoculture, quasi-native monoculture, and non-native co-culture grow at the same rate. This is an indication that PA01 isnt directly affected by the laser; the laser could be affecting the pJY209 which in turn changes the growth rate of PA01 when in a quasi-native co-culture.

4.1.4 Grouping data

Because experiments are require a large time investment and are prone to errors, we have to think about how to use all available data in order to have better statistics. Another analysis can be conducted by lumping monoculture and co-culture data together. Lumping the data together to just compare quasi- versus non-native states shows a difference in growth rates for both PA01 and pJY209, as shown in the following table. However, lag times are not significant.

	$1/\tau$		
	FAST	SLOW	FAST vs SLOW
PA01	0.0071±0.0021	0.0094±0.0027	**0.0280**
pJY209	0.0069±0.0028	0.0105±0.0033	**0.0015**

	Lag time		
	FAST	SLOW	FAST vs SLOW
PA01	104.22±81.76	66.71±61.45	0.2644
pJY209	86.33±110.02	77.39±104.51	0.8146

Though this is useful in achieving stronger statistics, this method has drawbacks in that information could be lost in the data. If monocultures and co-cultures are too different, results could be useless. However, this can be determined with more data.

High imaging rates are useful in studying mobility of cells and other individual cell dynamics. However, Population dynamics are largely affected by the species present and the imaging rate of the confocal microscope. There is a tradeoff between resolving unperturbed population dynamics and resolving individual cell dynamics that occurs with varying imaging rates. There is no universal imaging rate for biofilms. Imaging rate has a large effect on the measurements taken while observing biofilms, and those measures can change depending on the imaging rate. The effect of imaging should be studied before recording improper data.

4.2 Future Work

4.2.1 Second Exponential Phase

First, more data should be taken to strengthen the results. The data tended to have high standard deviations because of the numerous factors in biofilm development, and data will yield defined results. As can be seen in the table, some p-values are extremely close to the cutoff of 0.05 and could become significant results once more data is acquired.

In this experiment, we only studied the first exponential growth phase. Future analysis could include the second exponential growth phase to determine the cause of the second growth phase. It is possible that the cells suddenly enter another phase of growth, but we expect that the second exponential phase is a migration of biofilm mass from outside of the field of view. The biofilm develops quicker on the outside, causing bacteria to spill over into the field of view, as shown in the image below. The movement of bacteria into the field of view increases the voxel count, introducing a second exponential growth phase. This regime could give insight on bacteria motility or biofilm spread.

We expect that the cells are moving away from the field of view, growing slower in the field of view, or affected by both hypotheses. By observing individual cell doubling times, we can determine whether laser exposure is affecting the growth rate of the biofilm. On the other hand, if doubling rate analysis is not fruitful, still light can be shed on biofilm motility and cell influx.

The lack of effect on PA01 and pJY209 monocultures due to imaging rate was a surprise. However, this does not imply that a fast imaging rate has no effect on any biofilm growth. It is possible that a slow imaging rate already perturbs the biofilm

growth enough to elicit a decreased growth. It is possible that we did not use the proper times to quantify the scanning rate necessary to perturb the biofilm.

4.2.2 Corner Image Inconsistencies

The quantitative data shows that PA01 is not affected by laser growth. However, qualitative images of corners of the field of view as shown in Figure 3 show that the imaged region is affected by the laser. It is expected that a faster imaging rate would decrease the rate of growth of PA01 monoculture. However, quantitative results reveal no difference, and qualitatively, the field of view has more bright voxels than outside of the field of view. This qualitative analysis can be qualitatively evaluated by counting the voxels in a volume inside of and outside of the field of view and comparing the two.

The qualitative analysis could be a strange indication that laser exposure increases the growth rate of PA01. However, the increased brightness inside the field of view could also be explained by an increased rate outside of the field of view. As the brightness of the field of view increases, the brightness of the outside could be decreasing because it has formed a mat. The outside could form a mat faster than the inside because of the increased rate of growth. This hypothesis could be texted by viewing the biofilm under brightfield and measuring the depth. If the depth of the outside is thicker, that indicates that the biofilm is more developed and thus has a higher rate growth. This would imply a lower growth rate for PA01 under faster scanning.

Finally, the edge of the field of view has no growth. This is surprising, and this phenomenon can be explored in the future.

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